Atty. Dkt. No.

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE :

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN

(Attorney Docket No.)

the specification of which (check one)

___ is attached hereto.

__X__ was filed on **NOVEMBER, 05 2004** as United States Application Number or PCT International Application Number **PCT/IB2004/003882** and was amended on _____ (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's

certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?
0313261	**FRANCE**	**November 12, 2003**	**YES**	**NO**

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date

I HEREBY CLAIM the benefit under Title 35, United States Code, §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

I HEREBY APPOINT the registered attorneys and agents at Customer Number 22428

22428

22428

PATENT TRADEMARK OFFICE

to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith.

Atty. Dkt. No.

I request that all correspondence be directed to:

Stephen B. Maebius
FOLEY & LARDNER
Customer Number: 22428
22428
22428
PATENT TRADEMARK OFFICE

Telephone: (202) 672-5569
Facsimile: (202) 672-5399

I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Name of first inventor	**Hervé GROUX**
Residence	**BIOT - FRANCE**
Citizenship	**French**
Post Office Address	**13 chemin des Combes, 06560 LE ROURET - FRANCE**
Inventor's signature	
Date	
Name of second inventor	**Valérie BRUN**
Residence	**BIOT - FRANCE**
Citizenship	**French**
Post Office Address	**Résidence la pinède, 450 Avenue de St Philippe, 06410 BIOT - FRANCE**
Inventor's signature	
Date	05/02/07

Atty. Dkt. No.

Name of third inventor	**Arnaud FOUSSAT**
Residence	**BIOT - FRANCE**
Citizenship	**French**
Post Office Address	**Résidence la pinède, 450 Avenue de St Philippe, 06410 BIOT - FRANCE**
Inventor's signature	
Date	9/5/02/07 Date de Signature - SVP

Atty. Dkt. No. 065691-0443

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Hervé GROUX et al.
Title:	USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN
Appl. No.:	10/579,078
International Filing Date: 371(c) Date:	11/5/2004
Examiner:	Unassigned
Art Unit:	Unassigned
Conf. No.:	1413

CONSENT OF ASSIGNEE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

TXCELL, with an address of Route de Saint Antoine de Ginestière, Bâtiment Arc, Hôpital de l'Archet, 06200 Nice, France, which is the co-assignee of the above-identified application as evidenced by the attached copy of an Assignment document executed by two of the three listed inventors, hereby consents to the accompanying Petition to Correct Inventorship and supporting documentation, wherein non-signing inventor Hervé Groux is being included as an inventor of the above-identified patent.

The undersigned is empowered to sign this consent on behalf of the assignee.

Date: *May 23, 2007*

Name: Frederic HAMMEL

Title: CEO TxCell SA

Signature:

Sole or joint | USA Patent Appln | For Inventions made outside USA

ASSIGNMENT

In consideration of the sum of One Dollar ($ 1.00) and other good and valuable consideration paid to each of the undersigned, to wit:

1. ***Hervé GROUX***
2. ***Valérie BRUN***
3. ***Arnaud FOUSSAT***

the receipt and sufficiency of which are hereby acknowledged by the undersigned who at the behest of, hereby sell(s), assign(s) and transfer(s) unto,

1. ***TXCELL : Route de Saint Antoine de Ginestière, Bâtiment Arc, Hôpital de l'Archet, 06200 NICE – France***
2. ***INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM) : 101, rue de Tolbiac, 75013 PARIS - FRANCE***

(hereinafter designated "ASSIGNEE") the entire right, title and interest for the United States of America as defined in 35 U.S.C. 100, in the invention known as

USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN

For which an application for Letters Patent of the United States of America:

(a) has been executed by the undersigned on

(b) has been filed on
and assigned Serial No.

and the undersigned hereby authorize(s) and request(s) the United States Commissioner of Patents and Trademark to issue said Letters Patent to the said ASSIGNEE, for its interest as ASSIGNEE, its successors, assigns and legal representatives; the undersigned agree(s) that the attorneys of record in said application shall hereafter act on behalf of said ASSIGNEE;

AND the undersigned hereby agree(s) to transfer a like interest, upon request of the said ASSIGNEE, its successors, assignes and legal representatives, and without further remuneration, in and to any and all divisions, continuations, substitutes, and reissues thereof; and to testify and execute any papers for ASSIGNEE, its successors, assigns and legal representatives, deemed essential by ASSIGNEE to ASSIGNEE's full protection and title in and to the invention hereby transferred.

Signed on the date(s) indicated beside my/our signature(s)

INVENTOR(S) concurrently with application	**DATE SIGNED**	**WITNESS(ES)**
Hervé GROUX		
Valérie BRUN	5.02.07	05.02.07
Arnaud FOUSSAT	05.02.07	Alexandra CASTELAIN

Date & Signature

Atty. Dkt. No. 065691-0443

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Hervé GROUX et al.

Title: USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN

Appl. No.: 10/579,078

International Filing Date: 11/5/2004
371(c) Date:

Examiner: Unassigned
Art Unit: Unassigned

Conf. No.: 1413

CONSENT OF ASSIGNEE

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM), with an address of 101, rue de Tolbiac, 75013 Paris, France, which is the co-assignee of the above-identified application as evidenced by the attached copy of an Assignment document executed by two of the three listed inventors, hereby consents to the accompanying Petition to Correct Inventorship and supporting documentation, wherein non-signing inventor Hervé Groux is being included as an inventor of the above-identified patent.

The undersigned is empowered to sign this consent on behalf of the assignee.

Date: 23 MAI 2007

Name: DENISE HIRSCH

Title: directrice Pôle PI

Signature:

INSTITUT NATIONAL de la SANTE et de la RECHERCHE MEDICALE
101, rue de Tolbiac
75654 PARIS CEDEX 13

L'INSERM
Pour le Directeur Général
et par délégation

Sole or joint

USA Patent Appln

For Inventions made outside USA

ASSIGNMENT

In consideration of the sum of One Dollar ($ 1.00) and other good and valuable consideration paid to each of the undersigned, to wit:

1. ***Hervé GROUX***
2. ***Valérie BRUN***
3. ***Arnaud FOUSSAT***

the receipt and sufficiency of which are hereby acknowledged by the undersigned who at the behest of, hereby sell(s), assign(s) and transfer(s) unto,

1. ***TXCELL : Route de Saint Antoine de Ginestière, Bâtiment Arc, Hôpital de l'Archet, 06200 NICE – France***
2. ***INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM) : 101, rue de Tolbiac, 75013 PARIS - FRANCE***

(hereinafter designated "ASSIGNEE") the entire right, title and interest for the United States of America as defined in 35 U.S.C. 100, in the invention known as

USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN

For which an application for Letters Patent of the United States of America:

(a) has been executed by the undersigned on

(b) has been filed on
and assigned Serial No.

and the undersigned hereby authorize(s) and request(s) the United States Commissioner of Patents and Trademark to issue said Letters Patent to the said ASSIGNEE, for its interest as ASSIGNEE, its successors, assigns and legal representatives; the undersigned agree(s) that the attorneys of record in said application shall hereafter act on behalf of said ASSIGNEE;

AND the undersigned hereby agree(s) to transfer a like interest, upon request of the said ASSIGNEE, its successors, assignes and legal representatives, and without further remuneration, in and to any and all divisions, continuations, substitutes, and reissues thereof; and to testify and execute any papers for ASSIGNEE, its successors, assigns and legal representatives, deemed essential by ASSIGNEE to ASSIGNEE's full protection and title in and to the invention hereby transferred.

Signed on the date(s) indicated beside my/our signature(s)

INVENTOR(S) concurrently with application	**DATE SIGNED**	**WITNESS(ES)**
Hervé GROUX		
Valérie BRUN	5.02.07	05.02.07
Arnaud FOUSSAT	05.02.07 Date de Signature	Alexandra CASTELAIN

Statement of Facts in Support of Petition Under 37 C.F.R.§ 1.47(a)

I, Jacques Warcoin, hereby declare the following:

1. I am one of the European Patent Attorneys at Cabinet Regimbeau having the Power of Attorney to prosecute international application PCT/IB2004/003882, as evidenced by the attached Power of Attorney (Attachment A) for said international application signed by the three listed inventors, namely, Hervé Groux, Valérie Brun and Arnaud Foussat, filed in the name of Applicants: TXCELL and Institut National de la Sante et de la Recherche Medicale (INSERM).

2. Hervé Groux has refused to sign any and all documentation in relation to the U.S. Application No. 10/579,078, which is the US National Stage of international application PCT/IB2004/003882. In particular, he has refused to sign a Declaration and Power of Attorney form and an Assignment document.

3. Hervé Groux was the Director General of TXCELL from December 9, 2004 to September 7, 2005. In this capacity, Hervé Groux signed the attached Power of Attorney (Attachment B) on behalf of applicant TX CELL in international application PCT/IB2004/003882).

4. Hervé Groux also signed the attached Declaration of Inventorship (PCT/RO/101) (Attachment C) for international application PCT/IB2004/003882.

5. On January 17, 2007, I sent a letter to Mr. Groux forwarding a Declaration and Power of Attorney and an Assignment document for U.S. Application No. 10/579,078, as well as a copy of the International application as published (WO 2005/046729) (see Attachment D, with English translation and return receipt).

6. On February 19, 2007, I received a letter from Mr. Groux dated February 16, 2007 (see Attachment E, with English translation). In the fourth paragraph of said letter, Mr. Groux refuses to sign the Declaration and Power of Attorney and Assignment documents.

7. On April 17, 2007, my associate, Francis Ahner, sent a letter to Mr. Groux (see Attachment F, with English translation and return receipt) informing him that his February 16, 2007, letter was forwarded to TXCELL, forwarding a copy of Arnaud Foussat's response to Mr. Groux's February 16, 2007, letter (see Attachment G, with English translation of pertinent paragraphs, and a copy of FOUSSAT et al., The Journal of Immunology, 2003, p. 5018-5026,

attached to Mr. Foussat's response letter), and again asking Mr. Groux to sign the Declaration and Power of Attorney and Assignment documents for U.S. Application No. 10/579,078.

8. On May 24, 2007, Mr. Groux sent an e-mail to my associate Jessica Raicar, in which he continues to refuse to sign the Declaration and Power of Attorney and Assignment documents for U.S. Application No. 10/579,078 (see Attachment H, with English translation).

9. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issued thereon.

June 14, 2007 _____

Date

_____

Jacques Warcoin

ATTACHMENT A

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Corporation Treaty)

(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request):*

1/ GROUX Hervé
27, avenue des Colibris - 06410 BIOT / FRANCE

2/ BRUN Valérie
Résidence la pinède - 450 Avenue de St Philippe - 06410 BIOT / FRANCE

3/ FOUSSAT Arnaud
Résidence la pinède - 450 Avenue de St Philippe - 06410 BIOT / FRANCE

Hereby appoint(s) the following person as: ☒ agent ☐ common representative

Name and address
(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country)

Jean-Jacques MARTIN, Robert SCHRIMPF, Jacques WARCOIN
Francis AHNER, Christian TEXIER, Eric LE FORESTIER, Jean-Robert CALLON de LAMARCK
WITH THE RIGHT TO ACT TOGETHER OR SEPARATELY
CABINET REGIMBEAU
MARTIN, SCHRIMPF, WARCOIN, AHNER, TEXIER, LE FORESTIER, CALLON de LAMARCK
EUROPEAN PATENT ATTORNEYS
20, rue de Chazelles, 75847 PARIS CEDEX 17, FRANCE

to represent the undersigned before
☒ all the competent International Authorities
☒ the International Searching Authority only
☒ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN

Applicant's or agent's file reference: 346905 / D20884

International application number (if already available) PCT/IB2004/003822

Filed with the following Office: International bureau of World Intellectual Property Office
as receiving Office and to make or receive payments on behalf of the undersigned

Signature of the applicant(s) *(where there are several applicants, each of them must sign next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power).*

GROUX Hervé BRUN Valérie FOUSSAT Arnaud

Date: 09 DEC. 2004

PCT

POWER OF ATTORNEY

(for an international application filed under the Patent Corporation Treaty)
(PCT Rule 90.4)

The undersigned applicant(s) *(Names should be indicated as they appear in the request):*

TXCELL
Route de Saint Antoine de Ginestière,
Bâtiment Arc
Hôpital de l'Archet
06200 NICE
FRANCE

Hereby appoint(s) the following person as: ☒ agent ☐ common representative

Name and address
(Family name followed by given name, for a legal entity, full official designation. The address must include postal code and name of country)

Jean-Jacques MARTIN, Robert SCHRIMPF, Jacques WARCOIN
Francis AHNER, Christian TEXIER, Eric LE FORESTIER, Jean-Robert CALLON de LAMARCK
WITH THE RIGHT TO ACT TOGETHER OR SEPARATELY
CABINET REGIMBEAU
MARTIN, SCHRIMPF, WARCOIN, AHNER, TEXIER, LE FORESTIER, CALLON de LAMARCK
EUROPEAN PATENT ATTORNEYS
20, rue de Chazelles, 75847 PARIS CEDEX 17, FRANCE

to represent the undersigned before
☒ all the competent International Authorities
☒ the International Searching Authority only
☒ the International Preliminary Examining Authority only

in connection with the international application identified below:

Title of the invention: USE OF LIPOPEPTIDES FOR ACTIVATING T LYMPHOCYTES THROUGH THE SKIN

Applicant's or agent's file reference: 346905 / D20884

International application number (if already available) PCT/IB2004/003882

Filed with the following Office: International bureau of World Intellectual Property Office
as receiving Office and to make or receive payments on behalf of the undersigned

Signature of the applicant(s) *(where there are several applicants, each of them must sign next to each signature, indicate the name of the person signing and the capacity in which the person signs, if such capacity is not obvious from reading the request or this power).*

TXCELL
Géroux Hervé
Directeur Général

TxCell
SA au Capital de 586 390 €
55, allée Charles Victor Naudin
06410 BIOT - SOPHIA ANTIPOLIS FRANCE
Tél. 04 97 21 83 00 / Fax 04 93 64 15 80
RCS Nice B 438 941 206 - APE 731 Z

Date: 10 JAN. 2005

Box No. VIII (iv) DECLARATION: INVENTORSHIP (only for the purposes of the designation of the United States of America)

The declaration must conform to the following standardized wording provided for in Section 214; see Notes to Boxes Nos. VIII, VIII (i) to (v) (in general) and the specific Notes to Box No. VIII (iv). If this Box is not used, this sheet should not be included in the request.

Declaration of inventorship (Rules 4.17(iv) and 51*bis*.1(a)(iv)) for the purposes of the designation of the United States of America:

I hereby declare that I believe I am the original, first and sole (if only one inventor is listed below) or joint (if more than one inventor is listed below) inventor of the subject matter which is claimed and for which a patent is sought.

This declaration is directed to the international application of which it forms a part (if filing declaration with application).

This declaration is directed to international application No. PCT/..... (if furnishing declaration pursuant to Rule 26*ter*).

I hereby declare that my residence, mailing address, and citizenship are as stated next to my name.

I hereby state that I have reviewed and understand the contents of the above-identified international application, including the claims of said application. I have identified in the request of said application, in compliance with PCT Rule 4.10, any claim to foreign priority, and I have identified below, under the heading "Prior Applications," by application number, country or Member of the World Trade Organization, day, month and year of filing, any application for a patent or inventor's certificate filed in a country other than the United States of America, including any PCT international application designating at least one country other than the United States of America, having a filing date before that of the application on which foreign priority is claimed.

Prior Applications: .....

.....

I hereby acknowledge the duty to disclose information that is known by me to be material to patentability as defined by 37 C.F.R. § 1.56, including for continuation-in-part applications, material information which became available between the filing date of the prior application and the PCT international filing date of the continuation-in-part application.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name: GROUX Hervé

Residence: BIOT / FRANCE
(city and either US state, if applicable, or country)

Mailing Address: 27, avenue des Colibris
06410 BIOT / FRANCE

Citizenship: FRENCH

Inventor's Signature: .....
(if not contained in the request, or if declaration is corrected or added under Rule 26*ter* after the filing of the international application. The signature must be that of the inventor, not that of the agent)

Date: 09 DEC. 2004
(of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 26*ter* after the filing of the international application)

Name: BRUN Valérie

Residence: BIOT / FRANCE
(city and either US state, if applicable, or country)

Mailing Address: Résidence la pinède - 450 Avenue de St Philippe
06410 BIOT / FRANCE

Citizenship: FRENCH

Inventor's Signature: .....
(if not contained in the request, or if declaration is corrected or added under Rule 26*ter* after the filing of the international application. The signature must be that of the inventor, not that of the agent)

Date: 09 DEC. 2004
(of signature which is not contained in the request, or of the declaration that is corrected or added under Rule 26*ter* after the filing of the international application)

☒ This declaration is continued on the following sheet, "Continuation of Box No. VIII (iv)".

Continuation of Box No. VIII (i) to (v) DECLARATION

If the space is insufficient in any of Boxes Nos. VIII (i) to (v) to furnish all the information, including in the case where more than two inventors are to be named in Box No. VIII (iv), in such case, write "Continuation of Box No. VIII ..." (indicate the item number of the Box) and furnish the information in the same manner as required for the purposes of the Box in which the space was insufficient. If additional space is needed in respect of two or more declarations, a separate continuation box must be used for each such declaration. If this Box is not used, this sheet should not be included in the request.

Name: FOUSSAT Arnaud

Residence: BIOT / FRANCE
(city and either US state, if applicable, or country)

Mailing Address: Résidence la pinède - 450 Avenue de St Philippe
06410 BIOT / FRANCE

Citizenship: FRENCH

Inventor's Signature: ..... Date: 09 DEC. 2004

Translation of the registered letter with AR from Cabinet REGIMBEAU sent to H. GROUX (13, Chemin des Combes, 05560 LE ROURET, France) on January 17th, 2007

DOCUMENTS TO BE SIGNED BY THE INVENTORS

Title : LIPOPEPTIDES
In the name of : TXCELL/INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)
Our ref. : D20884 – SDE/JR

Dear Sir,

We return to you concerning the procedure relative to the above-referenced patent application.

You will find enclosed the Declaration and Power of Attorney in the USA as well as the Assignments in the USA and Canada, which we thank you to forward us in return after signature.

We stress that it is compulsory to sign the documents. Indeed, lack of signature of the enclosed documents could lead to potential invalidity of the patent applications derived from the above-referenced International application and consequently cause a considerable damage to the firm TXCELL, which may ask for compensation.

You will also find enclosed for your information a copy of the present application as published on May 26., 2005 under the No. WO 2005/046729.

Please forward us the signed documents before:

February 16th, 2007.

We stay at your disposal for any complementary information.

Sincerely Yours,

Jacques WARCOIN

Encl.: - Declaration and Power of Attorney (USA);
- Assignments (USA and Canada);
- Copy of the International application as published (WO 2005/046729).

CABINET REGIMBEAU

Propriété Intellectuelle

European Patent and Trademark Attorneys
Conseils en Propriété Industrielle

Robert SCHRIMPF
Jacques WARCOIN
Francis AHNER
Christian TEXIER
Eric LE FORESTIER
Jean-Robert CALLON DE LAMARCK
Marc LEVIEILS
Jérôme COLLIN
Franck TETAZ
Evelyne ROUX

BUREAU DE PARIS
Frédérique FAIVRE PETIT
Olivier LABEY
Isabelle THILL
Michel PERNELLE
Nicolas TORNO
Olivier PRATS
Axelle TRICHARD
Isabelle MENDELSOHN
Hélène CORRET
Claire MOUGET-GONIOT
Martine BLOCH-WEILL
Aymeric VIENNE
Marie AUDREN
Emmanuelle LEVY
Julie BRUN
Olivier CLAVEAU
Nadine ROCABOY
Thomas MOISAND
Barbara NICOL
Emmanuelle LIMOUZY
Anne BOGARD
Stephanie DOUARD
Aimée DADOUN
Magali TOUROUDE
Nicolas RUIZ
Clarisse LE SALVER
Yohann REBOUSSIN
Mélanie AZEVEDO
Cécile PUECH
Nicolas HAUTIER

BUREAU DE LYON
Frank TETAZ
Directeur Agence
Isabelle PINAUD
Lois HEIBLIG
Jean-Jacques BOUILLET
Lucile VERNOUX
Sylvain THIVILLIER

BUREAU DE RENNES
Daniel LE FAOU
Directeur Agence
Jean-Yves BRANGER
Françoise BOMER
Eric ROUSSEL

VEILLE ET DOCUMENTATION
Isabelle CLÉRY
Véronique CLOT
Virginie PROUTEAU
David BOURSEAUX

SERVICES ADMINISTRATIFS
Brevets - Isabelle HAY
Marques - Josiane AIZEL

COMPTABILITÉ
Catherine BOURDONNEC

Membre d'une association agréée.
règlement par chèque est accepté.

COPIE

Recommandé AR

Monsieur H. GROUX
13 chemin des Combes
06560 LE ROURET

Paris, le 17 janvier 2007

DOCUMENTS A FAIRE SIGNER PAR LES INVENTEURS
Demande de brevet d'invention PCT N° IB/2004/003882 du 5 novembre 2004
Pour : "LIPOPEPTIDES"
Titulaire(s) : TXCELL/INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)
Nos réf. : D20884 – SDE/JR

Monsieur,

Nous revenons vers vous concernant la procédure relative à la demande de brevet ci-dessus référencée.

Vous trouverez en pièce jointe de ce courrier le pouvoir/déclaration d'inventeurs aux Etats-Unis, ainsi que les documents de cession aux Etats-Unis et au Canada, que nous vous remercions de bien vouloir nous retourner signés.

Nous insistons sur le fait qu'il est obligatoire de signer les documents. En effet, le défaut de signature des documents ci-joints risque de rendre les demandes de brevet issues de la demande Internationale ci-dessus référencée potentiellement invalides et causer de ce fait un tort considérable à la société TXCELL dont elle devra éventuellement demander réparation.

Nous vous adressons également ci-joint pour information copie de la présente demande telle que publiée le 26 mai 2005 sous le n° WO 2005/046729.

Ainsi nous vous prions de nous retourner ces documents signés avant le :

16 février 2007.

Nous restons bien entendu à votre disposition pour tout renseignement complémentaire qui vous serait nécessaire.

Dans cette attente, veuillez agréer, Monsieur, l'expression de nos sincères salutations.

Jacques WARCOIN

PJ. : - pouvoir/déclaration d'inventeurs (Etats-Unis) ;
- cessions (Etats-Unis et Canada) ;
- copie de la demande Internationale telle que publiée (WO 2005/046729).

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En provenance de :

Monsieur Hervé Graff
13 Chemin des Cantes
06620 LE ROURET

Présentation le : 19/01/2007
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Signature du destinataire ou du mandataire (Précisez nom et prénom)

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FRAB

D20884 SDE

Renvoyer à l'adresse ci-dessous :

Cabinet Reguiba au.
Mme Stéphanie DUARD
20 Rue de Chazelles
75847 Paris Cedex 17

Translation of the registered letter with AR of Hervé GROUX and Françoise COTTREZ (13, Chemin des Combes, 05560 LE ROURET, France) sent to Jacques WARCOIN at Cabinet REGIMBEAU on February 16th, 2007

Dear Sir,

We have well received your letters of January 25., 23., 11. and 17., relative to the patents "Tr1/Atherosclerosis", "Food Antigen", "Lipopeptides" and "Cell Factory".

Concerning the patent "Tr1/Atherosclerosis", please find enclosed the documents signed as indicated in the documents that you sent us.

Concerning the patent "Food Antigen" we will return you the signed documents as soon as possible after you forward us (preferably by email in order not to delay the procedures: hgroux@laposte.net) the text corresponding to the patent because our signature reveals that we "review and understand the contents of the above-identified International application". Thus in order not to have any surprise, as it already happened in the past, we would like that the exact text corresponding to this patent be sent to us, which you will understand.

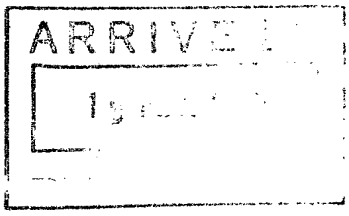
Concerning the patent "Lipopeptides" I cannot sign this patent as such. Indeed, the experiments which underly this patent were performed by M. Arnaud FOUSSAT and I realised *a posteriori* that it was not possible to have all the necessary trust in the scientific results which he presented. Thus I seriously doubt about the value of the results performed in this document. Consequently, in the absence of any experiments realised in a controlled manner by an independent laboratory, I will not give my support to this document.

Concerning the patent "Cell factory", I can only notice that there is no new element, despite an unfavorable report of the European authority and the contrary opinion that the Cabinet REGIMBEAU and ourselves formulated. Thus we can only repeat our position which is that in the absence of a dispensation of responsibility by which INSERM and TXCELL would be committed, we will not give our support to this document.

Sincerely Yours,

Hervé GROUX Françoise COTTREZ

Hervé et Françoise Groux
13 Chemin des Combes
06650 Le Rouret



à Cabinet Regimbeau
20 rue de Chazelles
75846 Paris Cedex 17
à l'attention de Mr Jacques Warcoin

SDE

Le Rouret, le 16 février 2007

Lettre recommandée avec AR

Cher Monsieur,

Nous avons bien reçu vos courriers du 25 janvier, 23 janvier, 11 janvier et 17 janvier relatifs aux brevets « TR1/ATHEROSCLEROSIS », « Food Antigen », « LIPOPEPTIDES » et « Cell factory ».

Concernant le brevet «Tr1/athérosclérose » je vous prie de trouver ci-joint les documents signés comme indiqué sur les documents que vous m'avez transmis.

Concernant le brevet « Food antigen » nous vous retournerons les documents signés dans les plus brefs délais dès que vous nous aurez transmis (par e-mail de préférence pour ne pas retarder les procédures : hgroux@laposte.net) le texte correspondant au brevet car notre signature révèle que nous avons « review and understand the contents of the above-identified international application ». Ainsi pour ne pas avoir de surprise, comme cela c'est déjà produit dans le passé, nous aimerions que le texte exact correspondant à ce brevet nous soit envoyé, ce que vous comprendrez.

Concernant le brevet « Lipopeptides » je ne peux pas signer ce brevet en l'état. En effet, les expériences qui sous-tendent ce brevet ont été réalisées par Monsieur Arnaud Foussat et j'ai pu réaliser à postériori qu'il n'était pas possible d'apporter toute la confiance nécessaire aux résultats scientifiques qu'il présentait. J'émets donc de sérieux doutes quant à la valeur des résultats présentés dans ce document. Aussi, en l'absence d'expériences réalisées de manière contrôlée par un laboratoire indépendant, je n'apporterai pas ma caution à ce document.

Concernant le brevet « Cell factory », je ne peux que constater qu'il n'y a pas d'élément nouveau, malgré un rapport défavorable de l'autorité européenne et l'avis contraire que le cabinet Regimbeau et nous mêmes avions formulés. Nous ne pouvons donc que redire notre position qui est qu'en l'absence d'une dérogation de responsabilité par laquelle l'INSERM et TxCell s'engageraient, nous n'apporterons pas notre caution à ce document.

Nous vous prions de croire, Cher Monsieur, à l'expression de nos salutations distinguées.

Hervé Groux

Françoise Cottrez

Translation of the Registered letter with AR from Cabinet REGIMBEAU sent to H. GROUX (13, Chemin des Combes, 05560 LE ROURET, France) on April 17th, 2007

DOCUMENTS TO BE SIGNED BY THE INVENTORS

Title : LIPOPEPTIDES
In the name of : TXCELL/INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)
Our ref. : 349659 D20884 – SDE/JR

Dear Sir,

We return to you concerning the procedure relative to the above-referenced patent family.

We forwarded your response of February 16, 2007 to the firm TXCELL.

You will find enclosed M. Arnaud FOUSSAT's response. Mr FOUSSAT considers that the suspicion which you expressed towards the results he obtained is unfounded.

Please note that we are going to forward among others the following documents to our Colleagues, for filing to the concerned Patent Offices, such that the procedure relative to the patent applications "*Lipopeptides*" in the USA and in Canada can be pursued :

- a copy of the article *FOUSSAT et al., The Journal of Immunology, 2003* cited by Mr FOUSSAT in his response;
- a copy of PCT Powers of Attorney where your signature appears as Legal Representative of the firm TXCELL, but also as co-Inventor (see enclosed copies for information). Your signature appears in particular beside those of Mrs BRUN and Mr FOUSSAT;
- a translation of our various exchanges with you, in particular our registered letters;
- a translation of your letter dated February 16, 2007 wherein you express reservations with respect to the relevance of Mr FOUSSAT's results;
- a translation of Mr FOUSSAT's response to your letter dated February 16, 2007.

Taking into account the situation and your previous involvement with respect to the PCT filing "*Lipopeptides*", it is in your interest to cooperate and to forward us the signed documents which we re-forward you as enclosed.

Sincerely Yours,

Francis AHNER

Encl.: - Mr FOUSSAT's response;
- PCT powers of Attorney signed by you (for information);
- Declaration and Power of Attorney (USA);
- Assignments (USA and Canada).

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06-40107-13-09-06

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D20884 SOE

Expéditeur

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LETTRE

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A CONSERVER PAR LE CLIENT

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Propriété Intellectuelle

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Conseils en Propriété Industrielle

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COMPTABILITÉ
Catherine BOURDONNEC

Membre d'une association agréée.
Le règlement par chèque est accepté.

Recommandé AR

Monsieur H. GROUX
13 chemin des Combes
06560 LE ROURET

Paris, le 17 avril 2007

DOCUMENTS A FAIRE SIGNER PAR LES INVENTEURS
Demande de brevet d'invention PCT N° IB/2004/003882 du 5 novembre 2004
Pour : "LIPOPEPTIDES"
Titulaire(s) : TXCELL/INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)
Nos réf. : D20884 – SDE/JR

Monsieur,

Nous revenons vers vous concernant la procédure relative à la famille de brevets ci-dessus référencée.

Nous avons communiqué votre réponse du 16 février 2007 à la société TXCELL.

Vous trouverez ci-joint la réponse de Monsieur Arnaud FOUSSAT. M. FOUSSAT considère que la suspicion que vous avez émise à l'égard des résultats qu'il a obtenus est sans fondement.

Nous vous prions de bien vouloir noter que nous allons communiquer à nos Confrères notamment les documents suivants pour dépôt aux Offices concernés afin que la procédure relative aux demandes de brevet « *Lipopeptides* » américain et canadien puisse continuer :

- copie de l'article *FOUSSAT et al., The Journal of Immunology, 2003* cité par M. FOUSSAT dans sa réponse ;

- copie des pouvoirs PCT portant votre signature en tant que Représentant Légal de la société TXCELL, mais également tant que co-Inventeur (cf. copies ci-jointes pour information). Votre signature apparaît notamment à côté de celle de Mme BRUN et de M. FOUSSAT ;

- traduction de nos différents échanges avec vous, en particulier nos lettres recommandées ;

- traduction de votre lettre du 16 février 2007 dans laquelle vous émettez des réserves quant à la légitimité des résultats de M. FOUSSAT ;

- traduction de la réponse de M. FOUSSAT à votre lettre du 16 février 2007.

Compte tenu de la situation et de votre engagement antérieur quant au dépôt PCT « *Lipopeptides* », il est dans votre intérêt de coopérer et de nous transmettre signés les documents que nous vous renvoyons ci-joint.

Dans cette attente, veuillez agréer, Monsieur, l'expression de nos salutations distinguées.

Francis AHNER

PJ. : - Réponse de M. FOUSSAT ;
- Pouvoirs PCT signés par vous (pour information) ;
- Pouvoir / déclaration d'Inventeurs (Etats-Unis) ;
- Cessions (Etats-Unis et Canada).

Cabinet REGIMBEAU
Stephanie Douard
20, rue de Chazelles
75847 PARIS Cedex 17

Sophia Antipolis le 25 avril 2007

Object :
Réponse à monsieur Groux concernant le brevet LIPOPEPTIDES (D20884 SDE)

Madame, Monsieur,

Je vous prie de trouver ci-dessous les détails relatifs à mes réponses concernant les allégations de Monsieur Groux concernant le brevet Lipopeptide

Point 1 :

Concernant le brevet «Lipopeptides » je ne peux pas signer ce brevet en l'état. En effet, les expériences qui sous-tendent ce brevet ont été réalisées par Monsieur Arnaud Foussat et j'ai pu réaliser à posteriori qu'il n'était pas possible d'apporter toute la confiance nécessaire aux résultats scientifiques qu'il présentait. J'émets donc de sérieux doutes quant à la valeur des résultats présentés dans ce document. Aussi, en l'absence d'expériences réalisées de manière contrôlée par un laboratoire indépendant, je n'apporterai pas ma caution à ce document.

Je réfute les propos de Mr. Groux sur ce sujet. Je me porte garant en tant qu'expérimentateur et inventeur pour les figures 1 et 2 dudit Brevet. Les résultats de ces expériences sont consignés dans des cahiers de laboratoires INSERM, ces expériences ayant été faites au sein de l'Unité 576, du Professeur A. Bernard à Nice. En ce qui concerne les figures 3, 4 et 5, c'est à Mr. Groux de prouver leur authenticité étant donné que ces résultats ne sont pas issus d'expériences que j'ai effectuées moi-même et donc absent de mes cahiers de laboratoires. Je m'étonne par ailleurs des remarques de Mr. Groux à mon égard ayant été sous sa responsabilité scientifique pendant plus de 5 ans. Les figures 1 et 2 du présent brevet sont notamment publiées dans un journal scientifique de renommée internationale (Foussat et al, The Journal of Immunology, 2003) et Mr. Groux en est dernier auteur donc responsable scientifique. Je m'étonne également de voir Mr. Groux réfuté aujourd'hui un Brevet qu'il a lui-même écrit, déposé et soutenu pendant plus de 3 ans.

English Translation
I totally disagree with the allegations of Mr. Groux. I certify as inventor having performed myself the experiments depicted in figures 1 and 2 of the patent. The results of these experiments are stored in lab books from the Inserm unit 576 of the Pr. A. Bernard, where these experiments have been performed. I also certify that the results depicted in the figure 3, 4 and 5 are not derived from my own experiments and are absents from my lab book. Mr. Groux should himself prove their authenticity. I am surprised of the allegations of

Mr. Groux concerning my own work since i was under his scientific responsibility during more than 5 years. Figures 1 and 2 of the patent are published in a scientific journal of international reputation (Foussat et al, The Journal of Immunology, 2003). Mr. Groux is the last author of this publication and thus is totally responsible for the published results. I' am also surprised to see Mr. Groux denying today a patent that he wrote himself, applied and defended during more than 3 years.

Points 2 :

Concernant le brevet « Cell factory », je ne peux que constater qu'il n'y a pas d'élément nouveau, malgré un rapport défavorable de l'autorité européenne et l'avis contraire que le cabinet Regimbeau et nous mêmes avions formulés. Nous ne pouvons donc que redire notre position qui est qu'en l'absence d'une dérogation de responsabilité par laquelle l'INSERM et TxCell s'engageraient, nous n'apporterons pas notre caution à ce document.

La discussion portant sur l'étape de purification des cellules produites à l'aide de l'usine cellulaire, je dois clarifier certains points :

- La pureté des produits de thérapie cellulaires comme de tout médicament injectable à l'homme est un élément crucial de sa qualité.
- Les résidus de l'usine cellulaire (ADN ou protéines) peuvent êtres nocifs pour l'injection aux patients et peuvent altérer tout utilisation ultérieure des cellules produites in vivo ou in vitro.
- L'utilisation de gradient de densités prédéfinies dans l'optique d'élimination de résidus des cellules de l'usine cellulaire est une nouveauté car c'est une solution trouvée au problème de la pureté et de la qualité du produit cellulaire. Notamment, ces gradients n'ont jamais été utilisés dans ce but, ni sur des préparations de lymphocytes et son différents des gradients utilisés de manière courante.
- La nouveauté réside également dans l'optimisation concomitante de 1) la pureté des cellules produites et 2) le rendement de récupération des cellules produites. Ce rendement fait également défaut lors de l'utilisation des gradients classiquement utilisés en séparation cellulaire.

Je vous prie, Madame, Monsieur, d'agréer mes sincères salutations

Arnaud Foussat
Directeur Scientifique TxCell Sa

The Journal of Immunology

A Comparative Study between T Regulatory Type 1 and $CD4^+CD25^+$ T Cells in the Control of Inflammation[1]

Arnaud Foussat,*[2] Françoise Cottrez,*[2] Valérie Brun,‡ Nathalie Fournier,* Jean-Philippe Breittmayer,* and Hervé Groux*[3‡]

There is now compelling evidence that $CD4^+CD25^+$ T cells play a major role in the maintenance of tolerance. Besides $CD4^+CD25^+$ T cells, different populations of regulatory $CD4^+$ T cells secreting high amounts of IL-10 (T regulatory type 1 (Tr1)) or TGF-β (Th3) have also been described in in vivo models. In the lymphocyte transfer model of inflammatory bowel disease, we show here that the control of inflammation during the first weeks is not due to a complete inhibition of differentiation of aggressive proinflammatory T cells, but is the result of a balance between proinflammatory and Tr cells. We also show that in the first weeks continuous IL-10 secretion was required to actively control inflammation. Indeed, treatment with anti-IL-10R Abs 3 wk after the start of the experiment completely reversed the protective effect of Tr cells. IL-10 secretion and control of inflammation could be provided by late injection of Tr1 cells that efficiently cure ongoing inflammatory responses in two different models of inflammation. In contrast, inflammation was not controlled when high numbers of $CD4^+CD45RB^{low}$ or $CD4^+CD25^+$ T cells were injected as early as 1 wk after the start of the experiment. These results confirm in vitro studies showing that $CD4^+CD45RB^{low}$ do not contain high IL-10-producing cells and suggest that $CD4^+CD45RB^{low}$ Tr cells maintain tolerance in vivo, in part indirectly, through the differentiation of IL-10-secreting Tr1 cells. *The Journal of Immunology,* **2003, 171: 5018–5026.**

Immune tolerance is maintained by several mechanisms that allow the immune system to discriminate between self and non-self. Moreover, the immune system is also exposed to repetitive stimulations by nonpathogenic Ags through inhalation and ingestion of foreign substances. To avoid chronic cell activation and inflammation, the immune system must develop unresponsiveness to such stimuli. Several mechanisms cooperate to maintain peripheral tolerance, including T cell anergy (1, 2), T cell depletion by apoptosis (3, 4), and active immune suppression (5, 6). Active immune suppression is mediated by specialized T cells whose function is to suppress the proliferation and activation of effector T cells. Recent studies have shown that regulatory T (Tr)[4] cells are contained within the $CD4^+$ T cell subset (7–9). However, a great deal of uncertainty remains about the lineages, differentiation factors, Ag specificity, and mechanisms of action of Tr cells. Moreover, several types of Tr cells have been described, each one with a unique mechanism of action that varies depending on the experimental model.

Recent studies have focused on a population of $CD4^+$ T cells that constitutively express the IL-2Rα (CD25). $CD4^+CD25^+$ T cells comprise ~5–10% of the peripheral T cell pool and exhibit immunosuppressive abilities both in vitro and in vivo. Taken together, all in vitro studies of murine and human $CD25^+$ T cells support a cell contact-dependent, cytokine-independent mechanism of suppression. Since suppression requires activation of $CD25^+$ T cells, it has been hypothesized that activation of these cells via their TCR induces a cell surface molecule(s) that mediates suppression by binding to a counter-receptor on the responder cells (10–12). However, it is not known how $CD4^+$ $CD25^+$ T cells execute their function in vivo, if they constitute only a small population of peripheral $CD4^+$ T cells (average, 6%) that needs direct cell contact as well as stimulation via the TCR to suppress unwanted T cell activation. In vitro studies usually employ high ratios of $CD4^+CD25^-/CD4^+CD25^+$ T cells, a situation that is hard to imagine in vivo. Moreover, in autoimmune diseases, IL-10, IL-4, and TGF-β have been implicated in the suppression mechanism of $CD4^+CD25^+$, in contrast to in vitro experiments (10, 13–17). This suggests that the regulation induced by $CD4^+$ $CD25^+$ T cells might be indirect and would require the differentiation of T cells secreting anti-inflammatory cytokines, as recently suggested (18).

We and others have reported that repetitive Ag-specific stimulations of $CD4^+$ T cells in the presence of IL-10 lead to the generation of another population of Tr cells (Tr1) predominantly producing IL-10 (19). We showed that these Tr1 cells have a regulatory function in vivo in a model of chronic inflammatory bowel disease (IBD) (7). In this model, injection of purified naive $CD4^+$ $CD45RB^{high}$ T cells from BALB/c mice into immunodeficient C.B-17 *scid* mice results in chronic inflammation in the colon. Colitis could be equally prevented by coinjection of $CD4^+CD45RB^{low}$ T cells, $CD4^+CD25^+$ T cells, or Tr1 cells (20, 21). Although the regulatory function observed with $CD4^+$ $CD45RB^{low}$ T cells could be restricted to the $CD4^+CD25^+$ T cell population (22, 23), the population of $CD4^+CD25^-CD45RB^{low}$ T cells always displayed some regulatory effects (22, 23). We therefore performed most of the experiments with the complete

*Institut National de la Santé et de la Recherche Médicale, Unité 343, Hopital de l'Archet, Nice, France; and †TxCell, Nice, France

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The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Association pour la Recherche sur le Cancer, the Ligue Nationale et Regionale de Lutte contre le Cancer, the Fondation pour la Recherche Médicale, and the Fondation Aupetit. A.F. and N.F. were recipients of an Association pour la Recherche sur le Cancer fellowship.

[2] F.C. and A.F. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Hervé Groux, Institut National de la Santé et de la Recherche Médicale, Unité 343, Route de St. Antoine de Ginestière, 06200 Nice, France. E-mail address: groux@unice.fr

[4] Abbreviations used in this paper: Tr, T regulatory; IBD, inflammatory bowel disease.

0022-1767/03/$02.00

$CD4^+CD45RB^{low}$ T cell population and controlled that the effects observed were similar to the effects obtained with the $CD4^+CD25^+$ T cell population.

In the present study using the same model of IBD we compared the mechanism of action of $CD4^+CD45RB^{low}$ T cells and Tr1 cells. We showed that although the protective effect of both $CD4^+CD45RB^{low}$ and Tr1 cells is prevented by blocking IL-10 function in vivo, only Tr1 cells display a curative effect when injected several weeks after the initiation of inflammation. These results support the hypothesis that the regulatory function of $CD4^+CD25^+$ T cells is mediated by the induction of anti-inflammatory Tr1 cell differentiation in vivo. Moreover, we confirmed the anti-inflammatory function of Tr1 cells in another model in which inflammation was induced in normal mice by repetitive applications of an irritant hapten.

Materials and Methods

Mice

Specific pathogen-free BALB/c and C.B-17 *scid* mice were obtained from CERJ (Le Genest Saint Isle, France). Homozygous DO11-10 mice were a gift from Dr. S. D. Hurst (DNAX Research Institute, Palo Alto, CA). Mice were maintained in our animal facility. C.B-17 *scid* mice were housed in microisolator cages with sterile filtered air (Rec Biozone, Margate, U.K.). Female mice were used at 8–12 wk of age.

Abs, media, and reagents

The medium used for T cell cultures was Yssel medium (24) supplemented with 10% FCS (Roche, Meylan, France) and 2×10^{-5} M β_2-ME (Invitrogen, San Diego, CA). Recombinant mouse IL-10 and IL-4 were gifts from Dr. R. L. Coffman (DNAX Research Institute, Palo Alto, CA). Recombinant mouse IFN-γ and IL-12 were purchased from R&D Systems (Minneapolis, MN). Purified anti-IL-4 (11B11), anti-IL-10 (2A5), anti-IFN-γ (XGM1.2), and biotin-anti-IL-4 (24G2), -anti-IL-10 (SXC1), and -anti-IFN-γ (R4-6A2; all from BD PharMingen, Le Pont de Claix, France) were used for cytokine assays. The following mAbs were used for mouse cell detection and purification: anti-I-A^d (AMS-32.1) anti-CD8 (53-6.7), anti-CD11b (M1/70), anti-B220 (RA36B2), FITC-conjugated anti-mouse CD45RB (16A), Tricolor- or PE-conjugated anti-CD4 (GK1.5), PE-conjugated anti-CD62L (Mel-14), FITC-conjugated anti-CD25 (7D4), FITC- or biotynylated-KJ-1.26 mAb revealed by PE-labeled streptavidin, and FITC- and PE-conjugated isotype control Abs (BD PharMingen). The following Abs were used in vivo: GL113 (isotype control, rat IgG1) and 1B1.2 (anti-mIL-10R; provided by Dr. K. Moore, DNAX Research Institute). For in vivo use, mAb were purified by column chromatography from tissue culture supernatants. The resulting Abs were >98% pure and contained <3 endotoxin units of endotoxins/mg of protein. Lysis buffer, $OVA_{323-339}$ peptide, OVA, and oxazolone were from Sigma-Chemie (Saint Quentin Fallavier, France). $OVA_{323-339}$ lipopeptide was purchased from Bachem (Voisin-le-Bretonneux, France).

Cell purification and cytofluorometry

$CD4^+$ T cell subsets were purified from the spleens of mice as previously described (7). Briefly, cells were depleted of $B220^+$, $Mac\text{-}1^+$, $I\text{-}Ad^+$, and $CD8^+$ cells by negative selection using sheep anti-rat Ab-coated Dynabeads (Dynal, Oslo, Norway). The remaining cells were labeled with FITC-conjugated anti-CD45RB (25 μg/ml) and PE-conjugated anti-CD4 (10 μg/ml) and separated into $CD4^+$ $CD45RB^{high}$ and $CD4^+$ $CD45RB^{low}$ fractions by two-color sorting on a FACStar SE (BD Biosciences, Le Pont de Claix, France). All populations were >98% pure on reanalysis.

T cell populations and T cell clones

The mouse T cell clones were obtained from DO11-10 mice after in vitro differentiation as previously described (7). Naive ($MEL\text{-}14^{bright}$) $CD4^+$, $KJ\text{-}1.26^+$ cells were stimulated for 3 wk repeatedly with $OVA_{323-339}$ peptide in the presence of IL-4 and anti-IL-12, IL-12 and anti-IL-4, or IL-10 for Th2, Th1, or Tr1 cells, respectively. The populations obtained were either used in vivo or cloned at one cell per well by cytofluorometry (FACSVantage SE; BD Biosciences) and stimulated with irradiated splenocytes (4500 rad) and OVA peptide. Clones were then expanded and analyzed for cytokine secretion after activation with APCs and OVA peptide (Table I). Selected clones were then expanded by stimulation with irradiated splenocytes and OVA peptide every 2 wk and were further expanded with IL-2 (R&D Systems; 10 ng/ml). T cell clones were used at least 10 days after the last stimulation.

Reconstitution of C.B-17 scid *mice with T cell subpopulations*

C.B-17 *scid* mice were injected i.p. with 100 μl of PBS containing sorted $CD4^+$ T cell subpopulations and different numbers of T cell clones as indicated. Anti-cytokine and control Abs were injected i.p. in PBS.

Microscopic examination of colons

Colons were removed from mice and fixed in PBS containing 10% formalin. Paraffin-embedded sections (6 μm) were cut and stained with H&E. Tissues were graded semiquantitatively from 0 to 5 in a blinded fashion. A grade of 0 was given when there were no changes observed. Changes typically associated with other grades are as follows: grade 1, minimal scattered mucosal inflammatory cell infiltrates, with or without minimal

Table I. *Cytokine profile of the different T cells used*[a]

Name	Type	IL-2 (pg/ml)	IL-4 (pg/ml)	IL-10 (pg/ml)	IFN-γ (ng/ml)
A-10-9	Tr1	<40	<50	1874 ± 217	65 ± 9
A-10-11	Tr1	<40	<50	1595 ± 184	42 ± 4
Nice-1	Tr1	<40	<50	1936 ± 502	38 ± 12
Nice-2	Tr1	<40	<50	1273 ± 298	51 ± 9
N10-7	Tr1	<40	<50	1659 ± 432	37 ± 7
N10-11	Tr1	<40	<50	1804 ± 394	41 ± 5
N10-23	Tr1	<40	<50	1493 ± 276	13 ± 3
N12-4	Th1	219 ± 42	<50	<75	73 ± 13
N12-8	Th1	275 ± 31	<50	<75	97 ± 10
N12-13	Th1	196 ± 54	<50	<75	84 ± 17
N4-2	Th2	<40	912 ± 81	305 ± 49	<0.2
N4-9	Th2	<40	1065 ± 103	287 ± 36	<0.2
N4-12	Th2	<40	715 ± 59	412 ± 67	<0.2
	Pop.Tr1	<20	112 ± 19	12865 ± 1457	5 ± 0.1
	Pop.Tr1	<20	86 ± 21	14945 ± 1065	2.8 ± 0.2
	Pop.Th1	513 ± 106	<40	<75	156 ± 4
	Pop.Th1	312 ± 95	<40	<75	124 ± 15
	Pop.Th2	<20	2321 ± 769	6378 ± 834	<0.2
	Pop.Th2	<20	998 ± 143	5241 ± 984	<0.2

[a] T cell clones and T cell populations were generated as described in *Materials and Methods*. T cells (10^6 cells/ml) were stimulated with OVA peptide (0.6 μM) and irradiated total splenocytes (2×10^6 cells/ml). Cytokines were analyzed by ELISA in culture supernatants collected after 48 h of culture. For the different T cell clones results represent the mean ± SD of three representative experiments of stimulation. For the T cell populations, the results represent the mean ± SD of triplicate measure ments of two representative experiments.

epithelial hyperplasia; grade 2, mild scattered to diffuse inflammatory cell infiltrates, sometimes extending into the submucosa and associated with erosions, with minimal to mild epithelial hyperplasia and minimal to mild mucin depletion from goblet cells; grade 3, mild to moderate inflammatory cell infiltrates that were sometimes transmural, often associated with ulceration, with moderate epithelial hyperplasia and mucin depletion; grade 4, marked inflammatory cell infiltrates that were often transmural and associated with ulceration, with marked epithelial hyperplasia and mucin depletion; and grade 5, marked transmural inflammation with severe ulceration and loss of intestinal glands.

Immunohistochemistry

Portions of colons were snap-frozen in liquid nitrogen and stored at −70°C. Frozen sections (5 μm) were cut and mounted on glass slides. They were thoroughly dried at room temperature for 1 h and fixed in acetone at 4°C for 15 min. Sections were stained by an immunoenzyme technique using the biotin-avidin-peroxidase system. Briefly, sections were washed in PBS for 5 min. Then sections were saturated with biotin and avidin (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and incubated with KJ-1.26-biotin or the control isotype. After washing for 5 min in PBS, the sections were incubated with streptavidin-peroxidase. After a final wash, peroxidase was developed with a diaminobenzidene vector staining kit (Vectastain; Vector Laboratories), which gives a brown color.

Contact sensitivity to oxazolone

Contact sensitivity to oxazolone was performed by applying 20 μl of a 50 mg/ml oxazolone solution in acetone/olive oil (4/1, v/v) epicutaneously on the right ear once a day for 3 days. The left ear received the vehicle only. Ear thickness was monitored every day. $OVA_{323-339}$ lipopeptide was diluted at 50 μM in olive oil. Mice were treated for 6 days by applying daily 20 μl of 50 μM $OVA_{323-339}$ lipopeptide or olive oil directly on the inflamed ear.

Cytokine assays

A sandwich ELISA was used to measure IL-4, IL-10, and IFN-γ. In brief, ELISA plates (Polylabo, France) were coated with the appropriate coating anti-cytokine mAbs in carbonate buffer and incubated at 4°C overnight. Plates were blocked for 30 min at room temperature with 150 μl of 20% FCS/PBS added to each well. Fifty microliters of diluted supernatants from in vitro stimulated $CD4^+$ T cells were then added to the plates and incubated overnight at 4°C. After a washing step, 50 μl/well of the biotinylated second-step Ab was added. Plates were incubated for 1 h at room temperature and washed. The enzyme conjugate (streptavidin-peroxidase) was then added to each well. Plates were incubated at room temperature for 1 h and washed, and 100 μl/well of substrate was added (1 mg/ml ABTS). Plates were read on an ELISA reader at a wavelength of 405 nm after color development (iEMS reader; Labsystems, Helsinki, Finland).

Results

Low doses of Tr1 cells prevent IBD, whereas Th1 and Th2 cells have no protective effect

In the IBD model of lymphocyte transfer into immunodeficient mice, it has been shown that the induction of inflammation in the colon is correlated with the presence of Th1 cells (25), whereas protection is induced by regulatory cells secreting IL-10 and TGF-β. However, the respective roles of Th1, Tr1, and Th2 cells in the regulation of inflammation has not been analyzed. To compare the in vivo function of Tr1 cells to Th1 and Th2 cells with the same antigenic specificity, SCID mice reconstituted with $CD4^+CD45RB^{high}$ T cells were treated with different amounts of OVA-specific T cell clones of the different subtypes (Table I) and fed with OVA (100 ng/ml) in their drinking water (Fig. 1) as previously described (7). As few as 2×10^4 cells were required for Tr1 cells to display protection, whereas no inhibition of colitis was observed with Th2 cells even at 2×10^6 cells/mouse, suggesting that Th2 cells are not involved in the negative regulation of inflammation in this model. As expected, a slight enhancement in disease score was observed with high doses of Th1 cells. To analyze whether the protective effect of Tr1 cells, compared with that of Th2 cells, was due to higher survival or expansion, we analyzed the number of cells recovered after the transfer into SCID mice reconstituted with $CD45RB^{high}$ T cells. All mice were fed OVA. Analysis of the number of $CD4^+KJ1\text{–}26^+$ cells on day 2 after the transfer revealed that few cells survived (<1% in the different populations). As expected, in reconstituted mice a marked expansion of Th1 cells was observed 4 and 8 wk after the transfer. Th2 cells also proliferated in vivo, but to a lesser extent. In contrast, for Tr1 cells, an expansion was observed 4 wk after the transfer, but these cells were not recovered after 8 wk, suggesting that their expansion is only transient and correlates with the intensity of inflammation. As a control, treatment of mice with the different clones in the absence of $CD4^+CD4RB^{high}$ T cells did not lead to

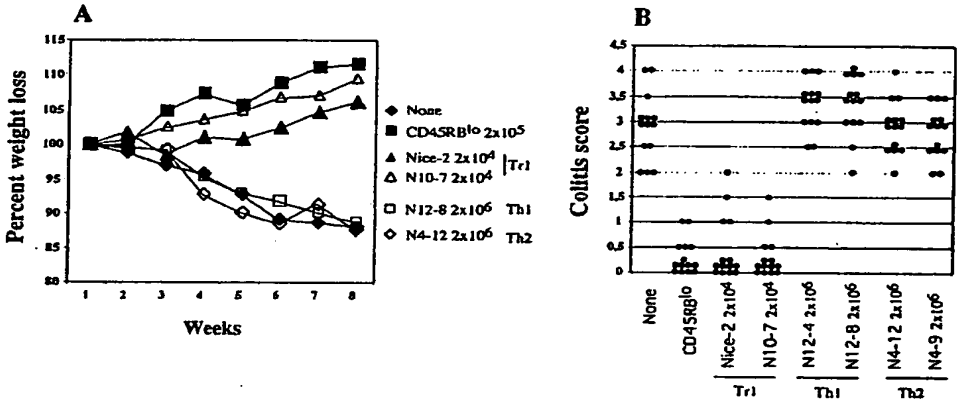

FIGURE 1. Tr1 cells specifically prevent IBD in vivo. *A*, C.B-17 *scid* mice were injected i.p. with sorted $CD4^+$ $CD45RB^{high}$ T cells (4×10^5 cells/mouse) and different numbers of the indicated OVA-specific T cell clones (Nice-2 and N10-7 are Tr1 cells, N12-4 and N12-8 are Th1 cells, and N4-12 and N4-9 are Th2 cells) or $CD4^+$ $CD45RB^{low}$ T cells (2×10^5 cells/mouse). T cell clones were injected at 2×10^4, 2×10^5, and 2×10^6 cells/mouse. Tr1 T cell clones were potent at 2×10^4 cells/mouse, and only that point is shown. Th1 and Th2 T cell clones did not prevent IBD even when injected in numbers as high as 2×10^6 cells/mouse, and only that point is shown. All mice received OVA protein (100 ng/ml) in their drinking water. Results represent the mean of seven mice per group of one representative experiment of two performed using these T cell clones. The experiment was repeated with other Tr1 (A-10-9, A-10-11, Nice-1, and Tr1 cell populations), Th1 (N12-13 and Th1 populations), and Th2 (N4-2 and Th2 populations) OVA-specific T cells, and similar results were obtained. *B*, Eight weeks after reconstitution, mice were killed, and colon pathology was graded. Data represent the colitis score of individual mice from two independent experiments.

colitis or significant expansion (not shown). These results suggest that Tr1 cells have a functional specificity in the control of inflammation.

Tr1 cell function in vivo requires IL-10

We have previously shown in vitro that IL-10 was required for both the function and the differentiation of Tr1 cells (7). Moreover, protection of IBD by regulatory $CD4^+CD45RB^{low}$ T cells has been shown to depend on the presence of IL-10 (15). To test whether IL-10 influences the function of in vitro-differentiated Tr1 cells in vivo, we used anti-IL-10R Abs to block the action of IL-10 in mice reconstituted with $CD4^+CD45RB^{high}$ T cells. Mice were treated weekly with anti-IL-10R or the isotype control mAb and, as shown in Fig. 2, *A* and *B*, treatment with anti-IL-10R abrogated protection from colitis induced by Tr1 cells or the $CD4^+CD45RB^{low}$ population, as all mice in these groups treated with anti-IL-10R developed colitis. Ab treatment alone did not enhance IBD induced by $CD4^+CD45RB^{high}$ cells (Fig. 2) or induced immune pathology in the absence of T cells, as unreconstituted recipients treated with anti-IL-10R did not develop colitis (data not shown). These results suggest that the secretion of IL-10 by regulatory T cells is crucial for the control of IBD.

In this type of reconstitution model of inflammation, the control of colitis, as observed with the transfer of Tr cells (either $CD4^+CD45RB^{low}$ T cells or Tr1 cells) could be due to an inhibitory effect of Tr cells on the function of activated Th1 effector T cells as well as to complete inhibition of effector T cell differentiation and expansion. To discriminate between these two possibilities, $CD4^+CD45RB^{high}$ T cell-reconstituted SCID mice were treated on day 0 with regulatory $CD4^+CD45RB^{low}$ T cells or Tr1 cells and Tr cell function was blocked with anti-IL-10R Ab treatment several weeks after the start of the experiment. As shown in Fig. 2, *C* and *D*, control of the inflammation leading to colitis requires continuous secretion of IL-10 during the first weeks after cell transfer. Indeed, inhibition of IL-10 function with anti-IL-10R treatment 3 wk after the injection of Tr cells completely reversed their protective effect. These results suggest that the protection of colitis is not due to complete inhibition of the differentiation of proinflammatory T cells directed against Ags present in the colon,

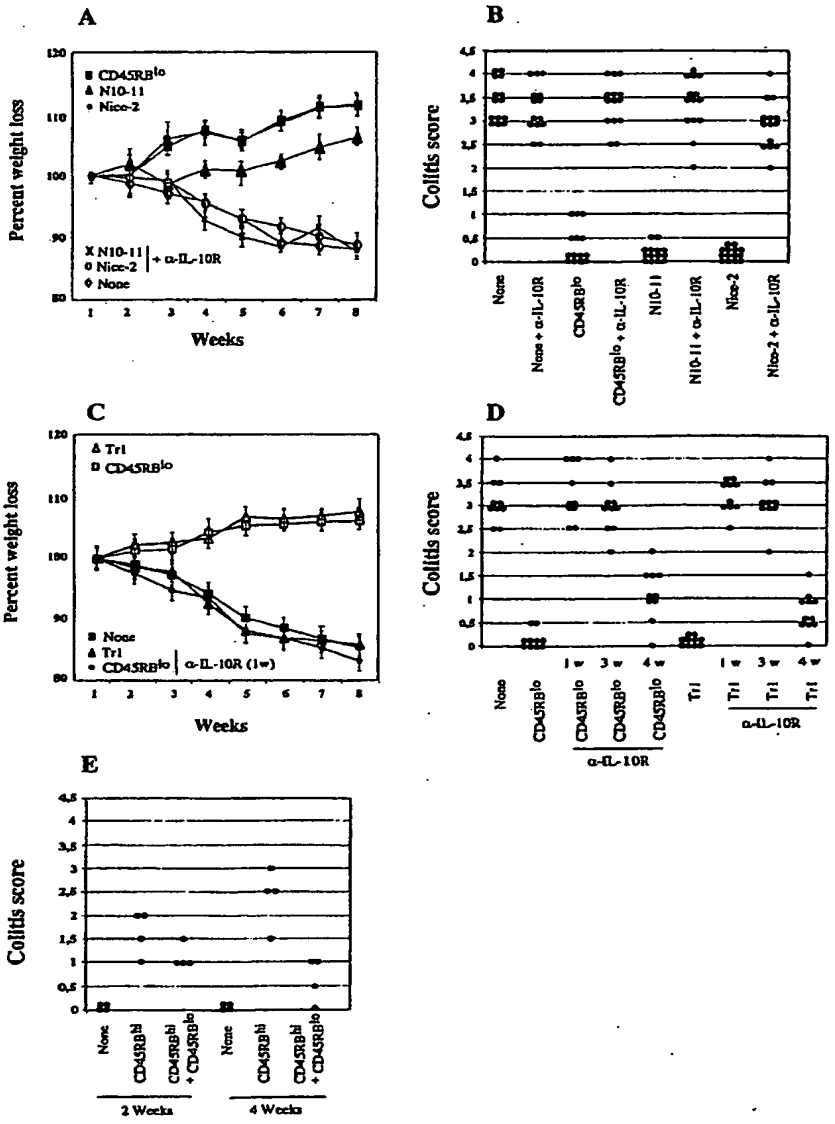

FIGURE 2. The presence of IL-10 is required for the function of Tr1 T cell clones. *A*, C.B-17 *scid* mice were reconstituted with $CD4^+CD45RB^{high}$ T cells (4×10^5 cells) and treated with $CD4^+CD45RB^{low}$ T cells (2×10^5 cells/mouse) or Tr1 clones (Nice-2 or N-10-11; 2×10^5 cells/mouse) on day 0. Mice were also treated with an isotype control Ab (GL-113) or with anti-IL-10 R Abs (1 mg/mouse on day 0, followed by a weekly treatment of 0.5 mg/mouse) as indicated. All mice received OVA (100 ng/ml) in their drinking water. Results represent the mean for seven mice per group of one representative experiment of two performed. Eight weeks after reconstitution, mice were killed, and colon pathology was graded (*B*). Data represent the colitis score of individual mice from two independent experiments. *C*, IL-10 is required several weeks after reconstitution. C.B-17 *scid* mice reconstituted with $CD4^+CD45RB^{high}$ T cells (4×10^5 cells/mouse) were treated with $CD4^+CD45RB^{low}$ T cells (2×10^5 cells/mouse) or a Tr1 cell population (2×10^5 cells/mouse) on day 0. One, 2, or 3 wk after reconstitution mice were also treated with an isotype control Ab (GL-113) or with anti-IL-10 R Abs (1 mg/mouse on the first day of treatment, followed by a weekly treatment of 0.5 mg/mouse) as indicated. All mice received OVA (100 ng/ml) in their drinking water. Results represent the mean for five mice per group of one representative experiment of two performed. Eight weeks after reconstitution, mice were killed, and colon pathology was graded (*D*). Data represent the colitis score of individual mice from two independent experiments. *E*, Tr cells do not completely prevent inflammation in the first weeks. C.B-17 *scid* mice reconstituted with $CD4^+CD45RB^{high}$ T cells (4×10^5 cells/mouse) were treated with $CD4^+CD45RB^{low}$ T cells (2×10^5 cells/mouse), and the colitis scores of the mice were analyzed 2 and 4 wk after cell transfer. Data represent the colitis scores of individual mice from two independent experiments.

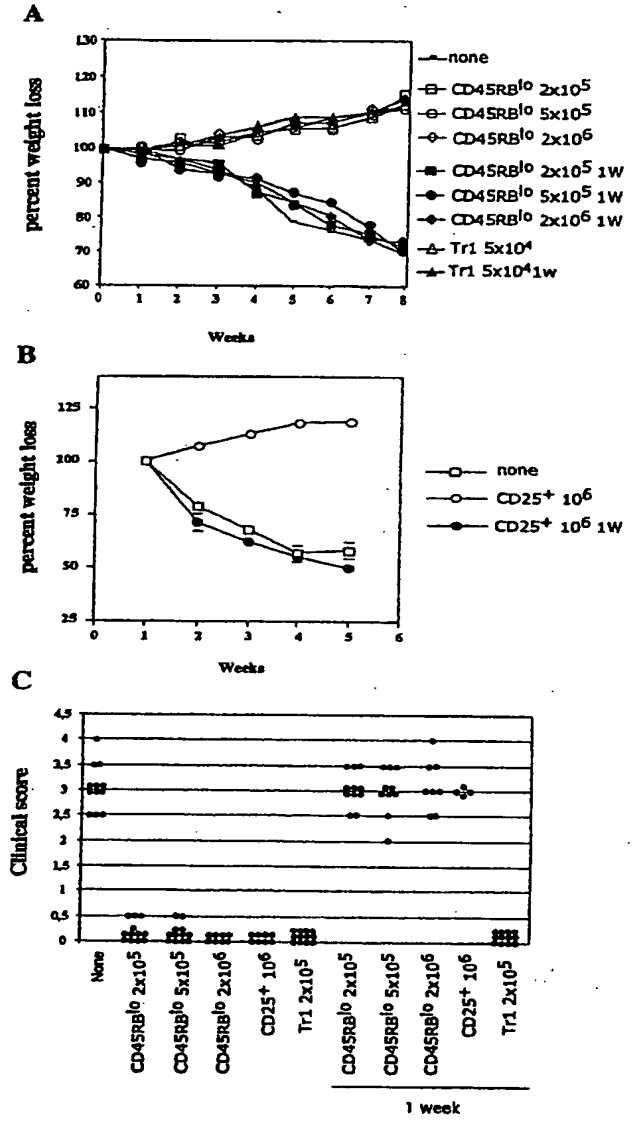

FIGURE 3. Tr1 cells, but not CD4+ CD45RB^{low} T cells, protect from IBD when injected after 1 wk. *A*, C.B-17 *scid* mice were reconstituted with sorted CD4+ CD45RB^{high} T cells (4×10^5 cells/mouse) alone and treated on day 0 or after 1 wk with different numbers of CD4+ CD45RB^{low} T cells or Tr1 cells (a population obtained after three weekly restimulations in the presence of IL-10) as indicated. All mice received OVA (100 ng/ml) in their drinking water. Results represent the mean for seven mice per group of one representative experiment of two performed. *B*, C.B-17 *scid* mice were reconstituted with sorted CD4+ CD45RB^{high} T cells (4×10^5 cells/mouse) alone and treated on day 0 or after 1 wk with 10^6 CD4+ CD25+ T cells as indicated. Results represent the mean of four mice per group of one representative experiment of two performed. *C*, At the end of the experiment, mice were killed, and colon pathology was graded. Data represent the colitis score of individual mice from two independent experiments.

but, rather, is the result of a balance between IL-10-secreting regulatory T cells and proinflammatory Th1 cells. The presence of proinflammatory T cells in the first weeks after reconstitution is confirmed by the histological analysis of colons of reconstituted mice 2 and 4 wk after cell transfer (Fig. 2*E*). These experiments show that mice reconstituted with both CD45RB^{high} and CD45RB^{low} populations display signs of inflammation in the colon, although to a lesser extent than mice reconstituted with only CD45RB^{high} CD4+ T cells, suggesting that in the first weeks, proinflammatory T cells are not yet completely controlled by regulatory T cells.

Differences between CD4+CD45RB^{low} T cells and Tr1 cells in the control of IBD

In this IBD model it has been clearly shown that the protective effect of CD4+CD45RB^{low} as well as CD4+CD25+ T cells is dependent on IL-10 secretion (15, 22). However, when activated in vitro with potent stimuli, these cells do not secrete IL-10 (10) (data not shown). Moreover, their inhibitory function in vitro is mediated by cell contact and is independent of IL-10 secretion. One could therefore hypothesize that CD4+CD45RB^{low} T cells do not have a direct inhibitory effect in vivo in this reconstitution model, but play their regulatory role indirectly by inducing the differentiation of IL-10-secreting Tr cells. To test this hypothesis, SCID mice reconstituted with CD4+CD45RB^{high} T cells were treated with a population of CD4+CD45RB^{low} T cells, a population of CD4+CD25+ T cells, or a population of Tr1 cells on day 0, simultaneously with CD4+CD45RB^{high} T cells or 1 wk after the onset of the experiment. In contrast to treatment with Tr1 cells, which remains effective after a delay of 1 wk (Fig. 3), treatment of SCID mice with CD4+CD45RB^{low} or CD4+CD25+ T cells is efficient only for the prevention of IBD, as injection of cells 1 wk after reconstitution failed to inhibit the development of colitis (Fig. 3*C*). We excluded the possibility that this absence of a protective effect of CD4+CD45RB^{low} or CD4+CD25+ T cells was due to low cell numbers in experiments performed with high doses (100 times the amount of cells required to show protection on day 0) of regulatory CD4+CD45RB^{low} or CD4+CD25+ T cells (Fig. 3), which again failed to display any inhibitory effect on colitis induction. We also excluded the possibility that the differences between Tr1 and CD25+ T cells in the inhibition of ongoing inflammation was due to a preferential expansion of Tr1 cells compared with CD4+CD25+ T cells. It has been shown (22) that CD4+CD25+ T cells expand in vivo and that 20–100% of the cells injected could be recovered 12–14 wk after their coinjection with CD45RB^{high} T cells. In contrast, the expansion of Tr1 cells was less effective, as <5% of the injected cells were recovered 4 wk after their transfer (Table II), and no cells could be found 8 wk after the transfer. Taken together, the absence of a curative effect, the absence of IL-10 secretion in vitro, and the requirement for continuous IL-10 secretion to control inflammation support the hypothesis that the CD4+CD45RB^{low} or CD4+CD25+ T cell population regulates colitis indirectly by inducing the differentiation of T cells that actively secrete immunoregulatory cytokines such as IL-10.

Tr1 cells cure ongoing IBD

To further analyze the ability of Tr1 cells to cure an ongoing inflammatory response, C.B-17 *scid* mice restored on day 0 with

Table II. *Survival and expansion of T cell clones in reconstituted SCID mice*[a]

	Th1	Th2	Tr1
2 days	0.3 ± 0.02	0.5 ± 0.1	0.1 ± 0.02
4 wk	102 ± 89	75 ± 51	14 ± 1.2
8 wk	125 ± 63	36 ± 21	ND

[a] SCID mice were injected with 10^6 in vitro differentiated Th1, Th2, or Tr1 cell populations and with 4×10^5 CD45RB^{hi} T cells. The animals were analyzed on day 2, 4, or 12 wk after cell transfer. The presence of CD4+KJ1-26+ (that specifically stained the injected T cell population) was analyzed in spleen, lymph nodes (axillary, inguial, and mesenteric), blood, and intestine. Data are expressed as the average ± SEM number of recovered cells ($\times 10^4$) in the different organs. ND, not detected

$CD4^+CD45RB^{high}$ T cells were transferred with Tr1 cells several weeks after the start of the experiment (Fig. 4, *A* and *B*). Even 6 wk after the transfer of pathogenic $CD4^+$ $CD45RB^{high}$ T cells, treatment of mice with Tr1 cells and OVA administration was followed by a remission of all inflammatory signs in the colon (Fig. 4*B*) associated with the recovery of the initial weight (Fig. 4*A*). In other experiments Tr1 cells were injected 4 wk after the reconstitution of mice with $CD4^+CD45RB^{high}$ T cells, and we analyzed the amount of Tr1 cells that infiltrated the colon as well as the extent of inflammation by immunohistochemistry. Analysis performed on colons 4 wk after the injection of $CD4^+$ $CD45RB^{high}$ T cells revealed an ongoing inflammatory response (Fig. 4*C*, *panel 1*). One week after the injection of Tr1 cell clones, several KJ-1.26$^+$ cells were detected within the inflamed colon (Fig. 4*C*, *panel 6*), and some signs of decreased inflammation were observed (Fig. 4*C*, *panel 2*). The inflammation quickly decreased within 3 wk (Fig. 4*C*, *panels*

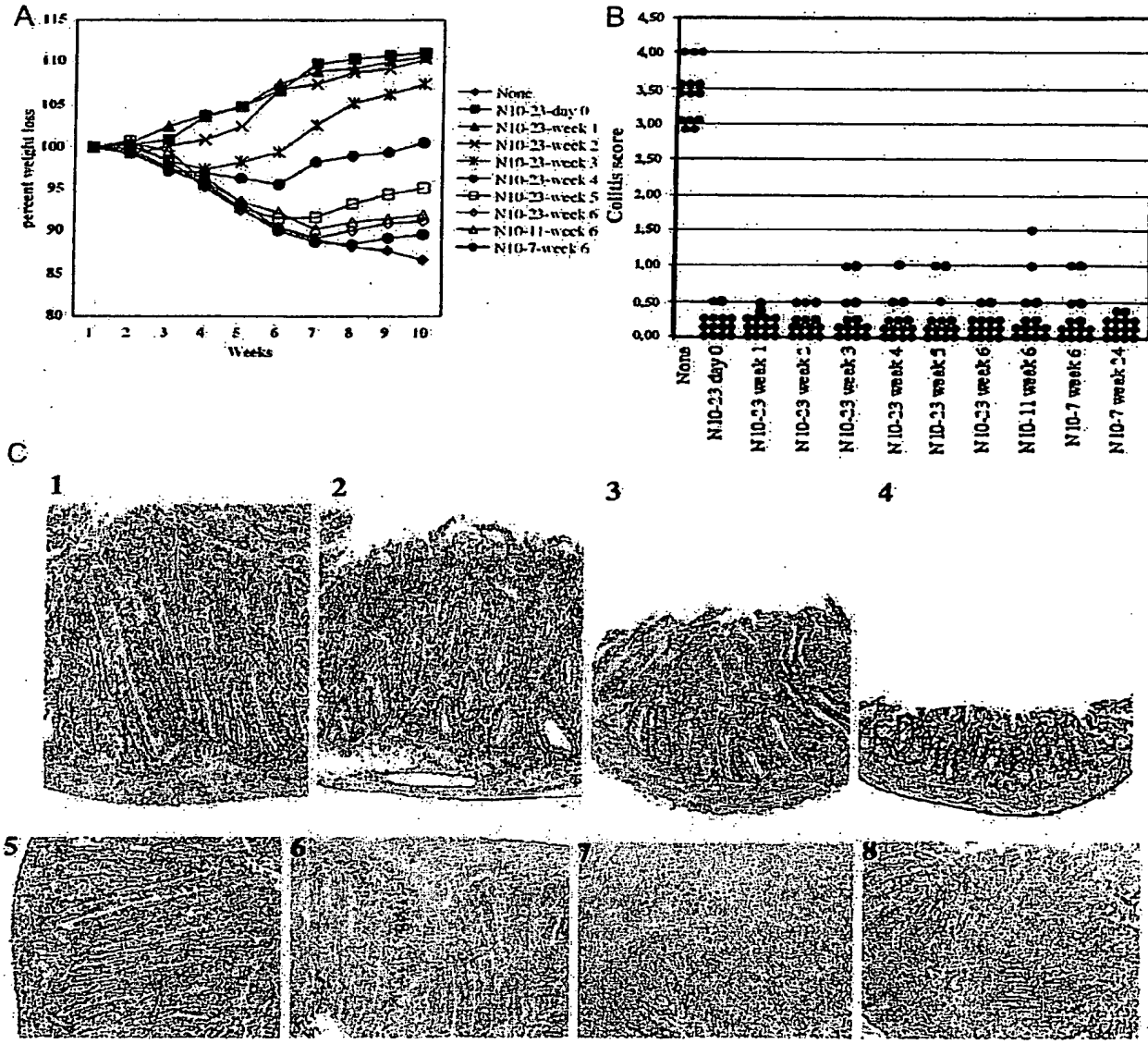

FIGURE 4. Tr1 cells cure ongoing inflammatory bowel disease. *A*, C.B-17 *scid* mice were reconstituted with $CD4^+CD45RB^{high}$ T cells and treated simultaneously or every week as indicated with 2×10^5 Tr1 cells. All mice received OVA (100 ng/ml) in their drinking water starting from the time of Tr1 cell transfer up to wk 10. Results represent the mean of seven mice per group of one representative experiment of two performed using these Tr1 cells. The experiment was repeated with other Tr1 (A-10-9, A-10-11, and Nice-1 T cell clones or Tr1 populations) OVA-specific T cells and gave similar results. *B*, Ten weeks after reconstitution, mice were killed, and colon pathology was graded. In some experiments mice received Tr1 cells at wk 6, were fed OVA for 4 wk, and were analyzed at wk 24 after cell transfer. Data represent the colitis scores of individual mice from two independent experiments. *C*, C.B-17 *scid* mice were reconstituted with $CD4^+CD45RB^{high}$ T cells (4×10^5 cells/mouse). Four weeks later, one group of mice was sacrificed, and their colons were examined (*panels 1* and *5*). High inflammation (grade 2–3) was observed. Mice were then treated with 2×10^5 cells/mouse of a Tr1 T cell clone (A-10-9) and fed OVA protein in their drinking water (100 ng/ml). Mice were sacrificed sequentially at 1 wk (*panels 2* and *6*), 2 wk (*panels 3* and *7*), or 3 wk (*panels 4* and *8*) after injection of the Tr1 clone, and the colons were analyzed for histology (*panels 1–4*) or by immunochemistry using a biotinylated KJ-1.26 mAb (*panels 6–8*). Numerous KJ-1.26$^+$ cells were detected in the inflamed colon 1 wk after injection of the clone (*panel 6*); 1 and 2 wk later, inflammation and numbers of KJ-1.26$^+$ cells progressively decreased (*panels 3* and *4*, and *panels 7* and *8*). After 3 wk (*panel 8*) only a few KJ-1.26$^+$ cells were detected.

3 and *4*), and the mice were completely cured 3 wk after the injection of Tr1 cell clones. In some experiments treatment with OVA was interrupted 4 wk after injection of Tr1 cells. In that case mice remained healthy for several months and displayed no sign of inflammation at the time of sacrifice several weeks or even months later (Fig. 4*B*).

Tr1 cells also exert an anti-inflammatory effect on skin irritation

To analyze the potential curative effect of Tr1 cells in a different inflammatory model, we set up skin inflammation experiments using the hapten oxazolone in three daily applications (Fig. 5A). Because Tr1 cells need to be activated at the site of inflammation, we first tested the ability of cutaneous application of lipo-OVA peptide to stimulate T cells. BALB/c mice were injected with naive OVA-specific DO11-10 T cells, and mice were treated for 6 days by applying daily 20 μl of 50 μM $OVA_{323-339}$ lipopeptide or olive oil directly to the ear. Analysis of T cells in the draining lymph nodes revealed an accumulation of activated ($CD25^+$) OVA-specific ($KJ1\text{-}26^+$) only in the ear draining lymph node treated with the lipopeptide (Fig. 5*A*). In that model, 3 days after the induction of ear inflammation with oxazolone, the mice were treated with OVA-specific Th1, Th2, or Tr1 T cell populations or a Tr1 cell clone (Fig. 5*B*), and the lipo-OVA peptide was applied for 6 days. In mice treated with Tr1 cells a marked decrease in inflammatory signs was observed, whereas treatment with Th1 or Th2 cells enhanced inflammation and edema (Fig. 5*B*). These results show that the specific regulatory function of Tr1 cells is not restricted to the colon, but is also efficient in different tissues and different types of inflammation, as previously reported by others (9, 26).

Discussion

We had previously shown that naive T cells, from OVA TCR-transgenic mice repeatedly stimulated with OVA and IL-10 result in the generation of T cell clones with a unique cytokine profile distinct from that of Th0, Th1, or Th2 cells (7). These Tr1 cells produce IL-10 and some IL-5 and IFN-γ, with or without TGF-β, but with little or no IL-2 and IL-4 production, and proliferate poorly following TCR-mediated activation. Functional studies of Tr1 cells directed against different Ags have shown that Tr1 cells have potent immunosuppressive properties and have been shown to prevent the development of Th1-mediated autoimmune diseases (7, 19, 27) and Th2 responses (28). Recently, in vitro manipulation with immunosuppressive drugs (26) or complementation of IL-10 with addition of IFN-α (29) or TGF-β (30) has facilitated the expansion of Tr1 cells.

Using defined polarized T cells sharing the same Ag specificity we showed in this manuscript that Tr1 cells specifically cure inflammatory responses. As previously shown in in vitro experiments (7), the protective effect of Tr1 cells in vivo is mediated by IL-10 secretion and is completely abrogated by the addition of anti-IL-10R Abs (Fig. 2). However, IL-10 secretion by itself is not sufficient to explain the protective effect of Tr1 cells, as Th2 cells, which also secrete IL-10, did not display any protective effect in the two different inflammatory models, IBD and skin inflammation. This lack of regulatory function of IL-10-secreting Th2 cells was not due to a preferential survival or expansion rate of Tr1 cells in vivo (Table II). However, the absence of inhibitory effects of Th2 in these models of inflammation could be due to the concomitant secretion of IL-4, a cytokine known to stimulate T cell functions, but other factors, such as different migration behavior and tissue localization in vivo, might also explain the specific function of Tr1 cells compared with Th2 cells.

It has been thoroughly demonstrated that $CD4^+$ $CD25^+$ T cells have important regulatory functions in vivo in rodents in the IBD model induced in immunosuppressed mice by the transfer of $CD4^+CD45RB^{high}$ T cells, it has been shown that the injection of $CD4^+CD45RB^{low}$ T cells prevents colitis (20), and that the regulatory function could be restricted to the $CD4^+CD45RB^{low}CD25^+$ population (22, 23). However, the $CD4^+CD45RB^{low}CD25^-$ population was also shown to display both a proinflammatory function when transferred alone and a regulatory function when coinjected with $CD4^+CD45RB^{high}$ T cells (22, 23). These results suggest that this cell population contains a mixture of proinflammatory cells as well as Tr cells that do not express the CD25 molecule. It was also demonstrated that $CD4^+CD25^+$ T cells inhibited the proliferative response of $CD4^+$ $CD25^-$ T cells in vitro (10). Moreover, a similar population of suppressor T cells with similar in vitro functions has been observed in humans (11, 12, 31). Numerous characteristics of $CD4^+$ $CD25^+$ T cells still need to be explained. One important question is how $CD4^+$ $CD25^+$ T cells execute their regulatory function in vivo, as they constitute only a small population of peripheral $CD4^+$ T cells (average, 6%) that need direct cell contact as well as stimulation via the TCR to suppress unwanted T cell activation. Moreover, in contrast to the lack of involvement of cytokines in $CD25^+$-mediated suppression in vitro (10, 13), IL-10, IL-4, and TGF-β have been implicated in mediating suppression in autoimmune diseases controlled by Tr cells in vivo (14–17). Recently, in vitro experiments using $CD4^+$ $CD25^+$ human T cells have suggested that the regulatory function of these cells might be supported in part by the differentiation of cytokine-secreting regulatory cells such as Tr1 or Th3 (18, 32). The data presented in this manuscript support that hypothesis. Here we showed that the control of inflammation is due not to a complete inhibition of proinflammatory cells in the first days after the transfer of Tr cells, but to a fine balance between proinflammatory and IL-10-secreting Tr cells. Indeed, some signs of inflammation were observed in the first weeks after the transfer of both proinflammatory $CD4^+$ $CD45RB^{high}$T cells and regulatory $CD4^+CD45RB^{low}$ T cells, suggesting that the regulatory cells actively control the inflammation. The importance of this balance, which is still active several weeks after the transfer of T cells, is demonstrated by the dramatic induction of colitis when IL-10 function is blocked 3 wk after the onset of the experiments. Therefore, the absence of a protective effect of the injection of high numbers of $CD4^+CD45RB^{low}$ (2 million cells) or $CD4^+CD25^+$ (1 million cells) T cells 1 wk after the reconstitution of SCID mice with $CD4^+CD45RB^{high}$ T cells suggests that the Tr cells secreting IL-10 are not contained in that population. This has been confirmed in a recent manuscript (33) in which the authors show that IL-10 is mandatory for the control of IBD, but when they injected $CD4^+CD25^+$ T cells isolated from $IL\text{-}10^{-/-}$ mice these cells still retained their ability to inhibit colitis induced by $CD4^+CD45RB^{high}$ T cells, demonstrating that IL-10 secretion by $CD4^+CD25^+$ T cells is not necessary to control IBD. Taken together, these results suggest that, at least in this model, the regulatory function of $CD4^+CD45RB^{low}$ or $CD4^+CD25^+$ T cells is in part indirect, by enhancing the differentiation of IL-10-secreting T cells. Recently, Mottet et al. (34) have reported that injection of $CD4^+CD25^+$ T cells inhibited ongoing colitis. This report is not discrepant with our results, as in both reports analysis of the colitis score 4–6 wk after the injection of $CD4^+CD25^+$ T cells did not reveal any marked beneficial effect, in contrast with the injection of Tr1 cells, which resulted in a rapid remission of inflammatory signs (Fig. 4). The curative effect of high number (10^6 cells) of $CD4^+CD25^+$ T cells was fully effective only after several weeks (10 wk), which again supports the hypothesis of an indirect mechanism in the control of inflammation mediated by $CD4^+CD25^+$ T cells.

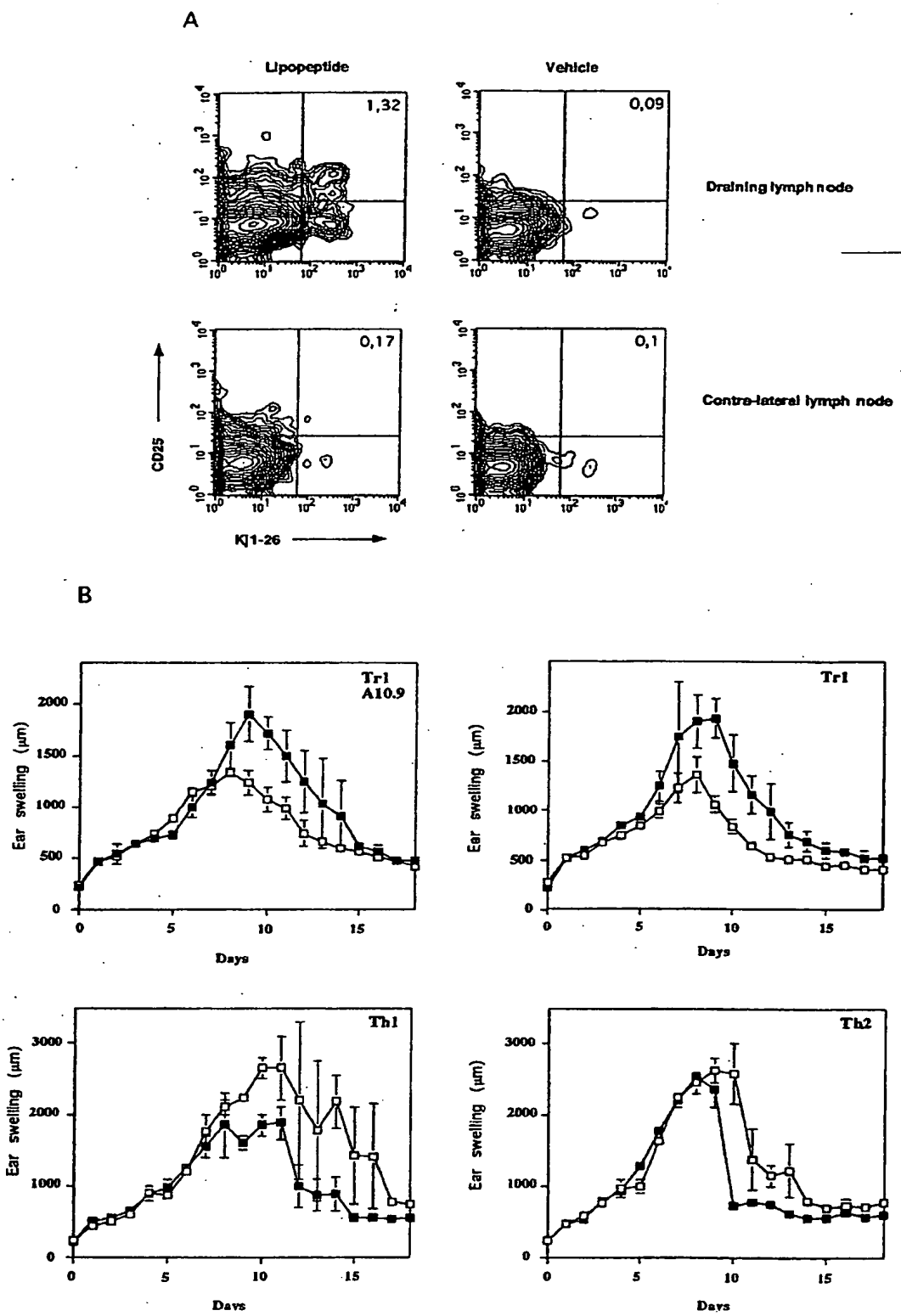

FIGURE 5. Tr1 cells accelerate the remission of hapten-mediated skin inflammation. *A*, Lipopeptide activates T lymphocytes in vivo. BALB/c mice were injected i.v. with 20×10^6 DO11-10 TCR-anti OVA transgenic splenocytes. Mice where then treated for 4 days by applying daily 20 µl of 50 µM $OVA_{323-339}$ lipopeptide or vehicle directly to one ear. On day 5 mice were sacrificed, and the draining lymph node and contralateral node cells were stained with the anti-Id KJ1-26 recognizing specifically DO11-10 T lymphocytes, anti-CD4, and anti-CD25 Ab. FACS analysis is shown for lymph node cells gated on $CD4^+$ T lymphocytes. *B*, BALB/c mice were treated with the hapten oxazolone (1 mg/ear) on days 0, 1, and 2. On day 3 all mice received 1 million Tr1, Th1, or Th2 T cells or the Tr1 clone (A-10-9) i.p. Mice were then treated for 6 days by applying daily 20 µl of 50 µM $OVA_{323-339}$ lipopeptide (□) or vehicle (■) directly to the inflamed ear. Results are shown as the mean ± SD thickness of inflamed ears for one representative experiment of two performed.

The differentiation of IL-10-secreting regulatory cells (Tr1) by $CD4^+CD25^+$ T cells could be mediated by direct cell contact, as suggested recently by in vitro studies using human $CD4^+CD25^+$ T cells (18) or by the action of $CD4^+CD25^+$ T cells on APCs. Indeed, it has been shown that the populations of dendritic cells in immunodeficient mice are completely different from those in normal mice and that the transfer of T cells restores the populations of dendritic cells (35). Further studies will be required to analyze whether the transfer of $CD4^+CD25^+$ T cells modifies dendritic cell populations to induce the differentiation of Tr1 cells.

In contrast to $CD4^+CD25^+$ T cells, we showed here that the injection of in vitro differentiated Tr1 cells, even 6 wk after the transfer of aggressive naive $CD4^+$ T cells, at the peak of the disease completely cures an ongoing inflammation. The regression of inflammation after the transfer of Tr1 cells was rapid and resulted in the complete absence of infiltrated leukocytes. Moreover, there was a surprising complete restoration of the damaged colon, which may be due to the combined action of TGF-β secreted by Tr1 cells and the rapid turnover of epithelial cells. The protective effect was not restricted to the colon, as Tr1 clones could effectively down-regulate inflammation in a skin model in which inflammation was mediated by an irritant hapten. Moreover, our results emphasize in both models the importance of local delivery of the specific Tr1 Ag to ensure its anti-inflammatory function. This requirement has also been shown by others in an experimental autoimmune encephalomyelitis model (26).

Taken together, our results support the idea that Tr1 and $CD4^+CD25^+$ regulatory T cells are two separate and specialized subsets of Tr cells; the latter have a central homeostatic function to regulate T cell proliferation through direct cell-cell contact mechanisms, and the former, endowed with anti-inflammatory capacity, infiltrate injured tissues to locally control inflammation through the release of IL-10 and TGF-β.

An important issue would be to isolate the natural counterparts of Tr1 cells differentiated in vitro. Preliminary studies using specific markers of these cells have shown that a decreased number of these natural Tr1 cells correlate with IBD in human (our manuscript in preparation).

Studies of Tr1 cells will soon offer some attractive avenues for potential therapeutic intervention. Indeed, they display several key advantages. They act through bystander suppression; thus, the pathogenic Ag that stimulates the aggressive T cells does not necessarily have to be known. Secondly, they preferentially migrate to inflamed organs and not to the sites of primary immune responses, thus preventing adverse side effects (our manuscript in preparation). Finally they require the presence of their specific Ag to be activated, providing a way to target them to a specific organ and to control their activation in vivo.

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Translation of the email dated May 24th, 2007 of H. GROUX

ATTACHMENT H

Good morning,

Relative to the « *Lipopeptide* » patent, I cannot content myself with Mr FOUSSAT's response. I have noted on several occasions, *a posteriori*, that I could not give the necessary trust to the results presented by Mr FOUSSAT. For some of them, after having asked to other laboratories to reproduce some of the data produced by Mr FOUSSAT, I have noticed that they were not reproducible. If I might have supported these results and submitted them for publication, this is because at this time, I thought that they were obtained in a rigorous way. I have doubts at the present time, that is why I am asking that these experiments be done again in a controlled manner. If these data were exact, it would not raise any problem and it could have already been done.

One more time, in the absence of these complementary data, I can only persist in my position.

Sincerely,

H. GROUX.

RAICAR Jessica

De: Hervé Groux [hgroux@laposte.net]
Envoyé: jeudi 24 mai 2007 17:01
À: RAICAR Jessica
Objet: Re: RAPPEL- URGENT :"LIPOPEPTIDES (D20884 SDE) - Signatures/Ajout d'inventeurs" - N/réfs.:E23876 SDE
Importance: Haute

Bonjour,

Concernant le brevet lipopeptide je ne peux pas me contenter de la réponse de Mr Foussat. J'ai pu constater à plusieurs reprises, à postériori, que je ne pouvait pas apporter toute la confiance nécessaire aux résultats présentés par Mr Foussat. Pour un certain nombre d'entre-eux, après avoir demandé à certains autres laboratoires de reproduire certaine des données produite par Mr Foussat j'ai pu constater qu'elles n'étaient pas reproductibles. Si j'ai pu soutenir ces résultats jusqu'à les soumettre à publication c'est qu'à l'époque je pensais qu'ils avaient été obtenus de façon rigoureuse. J'ai des doutes actuellement c'est pourquoi je demande à ce que ces expériences soit refaite de manière contrôlées. Si ces données sont exactes cela ne devrait pas poser de problème et cela aurait déjà pu être réalisé.

Encore une fois, en l'absence de ces données complémentaires je ne peux que persister dans ma position.

Sincèrement

H. Groux

Le 22/05/07 11:16, « RAICAR Jessica » <raicar@regimbeau.fr> a écrit :

CABINET REGIMBEAU
Intellectual Property
Conseils en Propriété Industrielle
European Patent and Trademark Attorneys
<http://www.regimbeau.fr>

Monsieur,

Nous revenons vers vous dans le cadre de l'objet cité en référence.

Nous vous prions de bien vouloir donner suite à notre courrier ci-joint.

Cordialement,

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informations contenues dans ce message électronique et tous les fichiers qui peuvent y être attachés est strictement interdit.